

Insights into functions of the H channel of cytochrome *c* oxidase from atomistic molecular dynamics simulations

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Abbreviations

CcO, cytochrome *c* oxidase; MD, molecular dynamics; QM/MM, quantum mechanical/molecular mechanical

Footnote: All amino acid numbering corresponds to the sequences of cytochrome *c* oxidase (CcO) from *Bos taurus*, unless otherwise stated.

Abstract

Proton pumping A-type cytochrome *c* oxidase (CcO) terminates the respiratory chains of mitochondria and many bacteria. Three possible proton transfer pathways (D, K and H channels) have been identified based on structural, functional and mutational data. Whereas the D channel provides the route for all pumped protons in bacterial A-type CcOs, studies of bovine mitochondrial CcO have led to suggestions that its H channel instead provides this route. Here we have studied H channel function by performing atomistic molecular dynamics simulations on the entire, as well as core, structure of bovine CcO in a lipid-solvent environment. The majority of residues in the H channel do not undergo large conformational fluctuations. Its upper and middle regions have adequate hydration and H-bonding residues to form potential proton-conducting channels and Asp51 exhibits conformational fluctuations that have been observed crystallographically. In contrast, throughout the simulations, we do not observe transient water networks that could support proton transfer from the N-phase towards heme *a* via neutral His413, regardless of a labile H-bond between Ser382 and the hydroxyethylfarnesyl group of heme *a*. In fact, the region around His413 only became sufficiently hydrated when His413 was fixed in its protonated imidazolium state but its calculated pK_a is too low for this to provide the means to create a proton transfer pathway. Our simulations show that the electric dipole moment of residues around heme *a* changes with the redox state, hence suggesting that the H channel could play a more general role as a dielectric well.

Significance statement

Cytochrome oxidase is a widespread respiratory enzyme that conserves energy released when oxygen is reduced by pumping protons across the membrane in which it is located. Here we use atomistic simulations of the whole bovine enzyme to investigate properties of the H channel, a structure that has been proposed to provide the pathway for pumped protons in mammalian forms of the enzyme. These studies show that, although parts of the structure could function in this manner, a gap persists. This gap could be bridged only if a buried histidine becomes protonated. Based on these simulations we propose that the H channel acts as a dielectric well, modulating effects of buried charge changes.

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Introduction

Cytochrome *c* oxidase (CcO) is a respiratory energy-transducing enzyme. It catalyzes electron transfer from cytochrome *c* to molecular oxygen, conserving the released energy as a charge and proton gradient across the membrane in which it is located (1). Mammalian mitochondrial CcOs are composed of at least 13 different polypeptides (2). They are members of a diverse ‘superfamily’ of homologous proteins (3-5), falling within the ‘A1’ subgroup, which includes many bacterial CcOs and quinol oxidases. All A1-type CcOs share a remarkably similar catalytic core formed by three subunits (I, II and III), which house the redox-active metal centres Cu_A, heme *a*, and a binuclear centre (BNC) composed of heme *a*₃, Cu_B and a catalytic tyrosine. Structures of several A1-type bacterial CcOs (6, 7) and of one mitochondrial form (bovine) (8) have been solved at atomic resolution (see Fig. 1). The electron transfer pathway and many details of the oxygen reduction chemistry have been resolved (9-11). However, although the basic principles of redox-coupled proton pumping are well established, the specific atomic details remain in dispute, particularly the roles of three possible proton transfer pathways (D, K and H channels) that have been identified in the A-type CcOs based on crystal structure data (9-11).

In bacterial CcOs, structural data, together with a wide range of biophysical and spectroscopic studies, strongly support a model for proton/electron coupling that involves only the D and K channels in internal proton transfers (9, 12, 13). The K channel delivers the first two substrate protons into the BNC. The D channel provides part of the route for the remaining two substrate protons and all four translocated protons. Experimental support for these proton-conducting functions has come in particular from studies of mutant forms of

bacterial CcOs (14). However, some significant issues remain. For example, in all CcO structures determined by X-ray crystallography, there is no connection for proton transfer from Glu242, the residue at the end of the D pathway, to either the BNC or to the likely exit route of the translocated protons. There is also the question of how the D channel could act as a conduit for both substrate and translocated protons. Unless gated precisely, the protons would always be driven into the BNC for exergonic water production, hence ‘short-circuiting’ the coupling process, rather than being driven across the membrane against a protonmotive force. One possible solution to these points has come from classical MD simulations that suggest that water molecules dynamically reorganise at different stages of the catalytic process to form a transient H-bonded link to either a proton trap above the hemes or to the BNC (15-18). Recent multiscale reactive MD simulations performed on the *Rb. sphaeroides* CcO structure have provided free energy profiles and predicted rates of internal proton transfer from the top of the D channel to both the proton trap and the BNC for wildtype (19) and decoupling mutants (20). Interestingly, internal proton transfer rates calculated from these simulations are found to be in good agreement with the kinetic data from electrometric experiments (21). Direct experimental evidence for functional water molecule reorganisation has come from FTIR studies of bovine and bacterial CcOs (22), though their location(s) within the channels remain unclear.

In contrast, the third hydrophilic H channel has been suggested to conduct translocated protons in bovine mitochondrial CcO, based upon structural and functional studies (8, 10). This H channel (Fig. 1, panel B) is separate from the BNC and the D and K channels. The lower part consists of a domain leading from mitochondrial matrix towards the hydroxyethylfarnesyl and formyl groups of heme *a*. It is proposed to open and become hydrated after the BNC has become reduced, creating a proton pathway for entry of four protons into a proton-collecting array close to a bound Mg^{2+} ion ‘above’ the BNC (23). Subsequent binding of oxygen closes this channel and one proton is proposed to be released into the P phase with each reduction/oxidation of heme *a* via an amide bond gate between Tyr440 and Ser441 (24) and an H-bonded network to Asp51 at the P phase surface (10). Support has come from structural perturbations induced by redox/ligand state changes in the water channel (25-28), the Asp51 residue (29) and the proposed proton-collecting site around the bound Mg^{2+} (23). These observations, together with effects of H channel mutations on coupling efficiencies in a chimeric bovine/human CcO construct (30, 31), have led to the proposal that these structures provide the route for translocated protons in mammalian mitochondrial CcOs. This contrasts with bacterial CcOs (32) where the H channel is less

complete, in particular lacking Asp51 and the YS residues whose amide linkage is proposed to form a gate. Furthermore, mutations introduced into the bacterial H channel are without effect (32), though some similar redox-induced crystallographic structural changes are observed in a serine residue that influences the opening of the bovine water channel (Ser382 in bovine enzyme) (33-35). It also contrasts with yeast (*Saccharomyces cerevisiae*) mitochondrial CcO:- this has a clear H channel (36) (though also lacking Asp51 and the proposed YS gate) but mutations within it do not affect proton translocation, whereas D-channel mutations induce effects similar to those induced in bacterial CcOs (37, 38). An alternative role of the H channel has been suggested in bacterial and yeast CcOs as a 'dielectric channel or well', providing groups that can cooperatively reorient their dipoles/charges in response to redox changes and hence modulate function (13).

Direct experimental testing of the functions of the H channel in mammalian forms of CcO is challenging, particularly because of the difficulties in introducing suitable mutations into the mitochondrially-encoded subunit I, and in direct kinetic observation of specific water and proton movements. Considerable advances have been made in molecular dynamics (MD) simulations of large protein structures embedded in aqueous media and/or membrane bilayers, including large coupled electron transfer complexes (39, 40). Such simulations, in conjunction with quantum mechanical (QM) calculations of metal center structures (41), have been successfully applied to regions of CcOs in order to predict oxygen reduction mechanism (42), water structure and protonic gating of the D channel in the vicinity of Glu242 (15, 18, 19, 43, 44) and behaviour of the K channel (45, 46). Here, we apply these methods to predict the dynamic properties of the H channel in an atomic model of membrane-embedded bovine CcO (see Models and Methods, and Tables 1-3) with focus on its possible role as a proton channel and/or a dielectric well.

Results

H channel dynamics and hydration

We first analyzed the dynamics and number of water molecules in the H channel region. Average numbers of water molecules were calculated over entire simulation trajectories by counting water oxygens within 5 Å of key H channel residues (subunit I residues His413, Ser461, Thr424, Ser454, Ser458, Arg38, Tyr440, Tyr441, Tyr371, and Tyr54, see Fig. 1). These results show that almost all water molecules within the entire H channel region exchange with the solvent during the simulation timescales but, in spite of that, their number does not change significantly throughout the simulations (Table 4). For all C1 simulations,

the average number of water molecules ranges from 20-22 with the sole exception of C1-VI (in which His413 was modeled in its cationic form, see Table 1). For the majority of C2 simulations, the number of water molecules in the H channel is found to fluctuate between 20 and 32, except for system C2-IV in which His413 was again modeled in its cationic form. To get further insights into these dynamics, we performed additional analyses by dividing the H channel into three regions: an upper region between the top of heme *a* and Asp51 that includes residues 54, 371, 440 and 441; a central region adjacent to heme *a* that includes residues 38, 424, 454 and 458; a lower region that includes residues 461 and 413 which spans Asp407 in the N phase and the central region (Fig. 1B).

Upper region of the H channel

We first considered the upper region of the H channel containing polar amino acid residues Tyr54, Tyr371, Tyr440 and Ser441. Structural data show that this region is relatively hydrophilic and contains the majority of the crystallographically-defined H channel water molecules (9 in fully oxidised, and 11 in fully reduced bovine CcO crystal structures 1V54 and 1V55, respectively (31)). Our simulations reveal additional hydration in this region (see Tables S1 and S4). Crystallographic (29) and FTIR (47, 48) data indicate that Asp51 is relatively buried and protonated in fully oxidised enzyme with its sidechain forming hydrogen bonds with Ser441 and Ser205 of subunits I and II, respectively (10). Accordingly, since all C1 simulations were performed with fully oxidised enzyme, all had Asp51 protonated. The hydrogen bond with the sidechain of Ser205 was found to be stable throughout these simulations (Table 5), in agreement with the structural and FTIR data, except for C1-I where the H-bond was found to be broken, leading to a larger average Asp51-Ser205 distance and recruitment of several additional waters into this region (Table S1).

FTIR data indicate that reduction of heme *a* and/or Cu_A leads to Asp51 deprotonation (47, 49). This is supported by the structure of the fully reduced enzyme which has been modelled with deprotonated Asp51 that loses its bonding to Ser205 and becomes more hydrated (10). For the majority of C2 simulations, performed on the experimentally characterized P-type catalytic states and various redox states of heme *a*/Cu_A, we modeled Asp51 in its deprotonated state (except C2-VI, see Table 2). In these cases, it was observed that the H-bond between Ser205 and deprotonated Asp51 is broken (Table 5) and Asp51 orients towards the solvent. This change in orientation of Asp51 is correlated with a large influx of water into the upper H channel region (Table 4), as also observed in the C1-I simulation described above in which the Asp51-Ser205 bond was again broken. Overall, the simulations are consistent with structural data that show that protonated Asp51 is relatively

buried and stabilized by H-bonding with Ser205, and that deprotonation leads to bond loss and hydration. This behavior of Asp51 and the high hydration of this region is in accord with the earlier suggestion (10) that there is a dynamic H-bond interconnectivity in the region that could facilitate proton transfer. However, the reorientation of Asp51 and its associated hydration increase do not induce any major conformational changes in the middle or lower parts of the H channel between heme *a* and the N phase where viable proton conduction pathways are less evident (see below).

It should be noted that the higher water occupancy in some C2 simulations (Table 4) results primarily from influx of water molecules into the hydrophilic upper region of the H channel in response to the loss of hydrogen bonding between deprotonated Asp51 and Ser205. The data in Tables S1-S6 using C1 and C2 systems further illustrate that the additional hydration associated with loss of the Asp51-Ser205 hydrogen bonding is primarily confined to the upper region, whereas variations in hydration in the middle and lower part of H channel are much smaller.

Middle region of the H channel

The middle section of the H channel, adjacent to heme *a*, potentially links Ser461 to the upper H-bonded and hydrated network. It contains polar amino acid residues and 4 buried water molecules in both oxidised (1V54) and reduced (1V55) crystal structures (Fig. 1). In all simulations, residues Arg38, Thr424, Ser454 and Ser458 remain stable in their crystallographic positions (see Fig. S1). The hydration level persists during the simulations and, due to the rigid structure of this region, the average water occupancy does not change much during the simulations in any of the states studied (Tables S2 and S5). The water molecules do exchange with solvent waters and their number does fluctuate in some states (Tables S2 and S5, see also Fig. 2), though the crystallographically-defined water sites remain fully occupied (Fig. 3). Analysis of simulation trajectories reveal that the bound water molecules are expelled by waters that transiently appear in the vicinity during the simulations, and the exchanges occur on a nanosecond timescale. Overall, the simulation data indicate that sufficient waters could appear in this region to create connectivity from S461 up to the upper H-bonded network. However, the only protonatable residue is Arg38 which is unlikely to deprotonate easily and most or all of any proton pathway would have to be provided by waters in that region.

Lower region of the H channel

The lower section links Asp407 in the N phase to the middle region *via* residues His413 and Ser461 (Fig. 1). His413, a partly conserved residue on which the H channel is named, is

located in a predominantly hydrophobic pocket with two crystallographically-defined water molecules above and below (Fig. 1, see also Fig. S8). However, in the crystal structure of fully oxidised enzyme there is a connectivity gap from there to Ser461, Ser458 and the two water molecules above them. The sidechain of nearby Ser382 (see Fig. 1) hydrogen bonds to the –OH of the hydroxyethylfarnesyl chain of heme *a*. In the fully reduced structure, however, this H bond is broken and Ser382 moves such that a cavity is formed that has been proposed to allow water to enter and hence allow proton transfer from the N phase via His413 into the middle region (23, 26). Indeed, in C1 simulations carried out with the oxidised form of CcO, the hydrogen bond between the -OH groups of Ser382 and the hydroxyethylfarnesyl chain of heme *a* persists, as do the two closest waters that are above Ser461/Ser458 (Fig. 4). For C2 simulations this hydrogen bond tends to dissociate in both P-type catalytic intermediate states or with the BNC reduced (Fig. 4). Hence, although this dissociation is consistent with the structure of the FR state, the data show that dissociation of the H-bond is not specifically correlated with any significant additional hydration of the cavity between His413 and Ser461. In all simulations that failed to increase hydration in this region, the common feature instead was that all had His413 in its neutral state; in all of these the low hydration persists and the occupancy of the two water sites above and below His413 do not fluctuate much (see snapshots in Fig. 3 panels A and C), including in those cases where the BNC metals are reduced (C2-VII and C2-VIII). To further test this behaviour, two $\sim 1 \mu\text{s}$ MD simulations were performed in the fully reduced (FR) state with the C3 system (Table 3). Again, hydration remained minimal in the regions immediately above and below His413 in these long time scale simulations, with only 1.6 ± 1.1 and 1.3 ± 0.8 water molecules within 5 Å of His413 and Ser461 in two simulation replicas. These conclusions are also supported by data in Fig. 5 and Tables S3 and S6, which show that this low water occupancy persists throughout the simulation times in all redox states studied for both C1 and C2 systems, provided that the His413 is neutral. This suggests that there is a permanent barrier to proton transfer in this region, regardless of the Ser382 conformation, in contrast to proposals based on structural and FTIR data. These findings agree with observations in *Rhodobacter sphaeroides* CcO (33) where the same type of structural change of the equivalent serine has been observed. In this enzyme the H channel is not operative, and recent kinetic data on wild-type and a Ser425Ala mutant show no major differences in their reaction kinetics or coupling efficiencies (50).

A low pK_a of His413 is in accord with a location that is surrounded by hydrophobic residues (Fig. S2). However, if the BNC is oxidised, heme *a* does display a weak E_m/pH

dependency over a wide pH range (51, 52), consistent with weak redox Bohr effects on one or more groups. It was shown that this is linked to a small proton uptake from the inner (51) or both (53) sides of the membrane upon reduction of heme *a*. Given its location, one of these groups might conceivably be His413 which, if it became protonated, might influence the surrounding region and its hydration level. In order to explore this further, we firstly calculated the pK_a of His413 by performing continuum electrostatic calculations on simulation snapshots in different redox states of the enzyme. We observed that the reduction of heme *a* causes an increase in the pK_a of His413 of about 1.5 pK_a units, but with values still too low ($pK_a < 5$) to result in significant protonation. This viewpoint is also supported by two independent computational approaches (see Models and Methods).

Nevertheless, we simulated the effect of protonating His413 (total charge +1) on the occupancy and dynamics of water molecules in the lower part of the H channel (Tables 1 and 2). In both C1 and C2 simulations, forced protonation of His413 does indeed induce more water molecules to appear in its vicinity, providing connectivity between the N-side of the membrane and His413, and between His413 and Ser461 (Figs. 3 and 5, and Tables S3 and S6), in sharp contrast to the case of neutral His413, in which no such water wires form (Fig. 3, compare panels A and C with B and D). However, recalculation of its pK_a using snapshots from simulations with protonated His413 again indicated predicted pK_a s (< 5) too low to support any significant His413 protonation with a concomitant increase in hydration. Hence, these data tend to rule out the opening of the channel by this effect.

As an additional test of the stability of hydration in the lower region of H channel, eight short MD simulations (three of setup C1-I and five of C3-II) were performed starting with a fully hydrated H-channel and a neutral His413. In all of these, water occupancy in the lower part of H channel rapidly declined within the first 5-20 ns (Figs. S3 and S4), strongly supporting the notion that hydration above and below neutral His413 is not stable. Based on C1-I simulations, we also reconstructed the free energy profile associated with the wetting of the lower part of the H channel (Fig. S5) using the Dynamic Weighted Histogram Analysis (54). These results further confirm that, when neutral, His413 destabilizes hydration in the lower H channel by at least 2-3 kcal/mol.

Given the importance of charge and position of a proton in hydration and in proton translocation through this pathway, we did separate classical and quantum mechanical/molecular mechanical (QM/MM) MD simulations in which a proton was treated explicitly (see Models and Methods). Firstly, data from multiple independent classical simulations show that an H_3O^+ ion is unstable in the H channel, in particular in the lower

segment between His413 and Asp407. In all five independent simulations, the H_3O^+ ion exited the region within first 15 ns of simulation time to the bulk aqueous phase at the N side of the membrane. A similar scenario was observed when QM/MM MD simulations were performed by simulating an explicit proton on water molecules connecting His413 to Asp407 (Fig. S2). Only when the proton was modeled on the water H-bonded to His413 did it form a doubly protonated His413, whereas in various other cases, proton rapidly diffused towards Asp407 (already during initial energy minimization, see also supplementary Video S1). Interestingly, in a relatively longer 15 ps QM/MM MD simulation of protonated Asp407, the proton never escaped the latter residue. In fact, the water chain connecting protonated Asp407 to His413 disassembled (see Video S2) in agreement with classical simulations.

Overall the data from multiscale simulations suggest that proton uptake from the N side of the membrane *via* His413 is strongly unfavourable because of both kinetic (absence of water wire) as well as thermodynamic (low pK_a of His413) reasons.

Redox coupled changes

Based on the simulation data presented above, the lower half of the H channel is unlikely to provide a pathway for coupled proton translocation. However, it may be expected that redox change of heme *a* is accompanied by some structural or hydration rearrangements in its environment induced by the net charge change (13). Comparisons of simulation data of states in which heme *a* is oxidised or reduced do not show any major differences in the dynamics of residues or hydration in the region next to heme *a*. In addition, as shown above, a redox Bohr protonation of His413 appears to be very unlikely. In order to explore more subtle possible redox-coupled changes that may occur in this region, we calculated the electric dipole moment of the sidechains of selected amino acid residues close to heme *a* (residues Tyr54, Tyr371, Gln428, Arg38, Ser454, Ser458, Thr424, Ser382 and Ser461). The data from multiple independent simulations in different redox states show that reduction of heme *a* (compare C2-I vs C2-II, and C2-VII vs C2-VIII) is coupled to reduction in the magnitude of electric dipole moment of nearby residues (Table 6, see also Fig. S6), consistent with the net charge change in the iron/heme system from +1 (oxidised) to 0 (reduced). Although polarization effects such as charge fluctuations are not accounted for in the simulations reported here, both C1 and C2 systems give roughly the same magnitude for the oxidised state and the subtle difference in electric dipole moment in different redox states is consistently observed in the C2 simulations. Such changes are to be expected when a net charge change occurs on a buried group and are consistent with a general function of part or all of the H channel structure as a ‘dielectric well’ (13) where small concerted changes in

polarizable groups occur to counteract the buried charge change.

Discussion

We have performed fully atomistic classical MD simulations on the entire structure of CcO and on various protonation and redox configurations on smaller 3-subunit model, with a focus on the possible role of the H channel in redox-driven proton pumping. Data from multiple independent simulations on both systems show consistent behavior in the main elements of the H channel, much of which is consistent with crystallographic data. Overall, there are no large scale conformational changes apart from those involving Asp51 and Ser382 that have been described in structural work. Here, for clarity of discussion, we have divided the H channel into three functional regions.

The upper region connects the top of heme *a* with Asp51 at the P phase border and includes residues Tyr54, Tyr371, Tyr440 and Ser441. Crystallographic data show that this region is hydrated and simulations indicate additional hydration, especially if Asp51 becomes deprotonated, a state in which it tends to move out of its relatively buried location (29). FTIR data (47, 49) have suggested that deprotonation is linked to heme *a*/Cu_A reduction, though this is surprising since it is the opposite of a classical redox-linked Bohr effect (55). It is also inconsistent with the -20 to -30mV/pH dependency of heme *a* redox potential when the BNC is oxidised (51, 52) and even weaker pH dependency when the BNC is reduced and CO-ligated (56). This anomaly is yet to be explained. Nevertheless, both the structural data and the simulations performed here suggest that ample hydrated pathways are available in this part of the structure to form possible proton relay pathways. Hence, it seems feasible that this region could indeed form a potentially gated exit pathway to the P phase for protons accumulated in the proton trap region.

The middle region of the H channel adjacent to heme *a* includes residues Ser458, Arg38, Ser454, Gln428, Tyr371 and Tyr54. As shown here, these residues stay more or less stable throughout the simulations. The few waters observed in crystal structures remain and their number even increases somewhat in the simulations. Hence, though a relatively rigid region and with Arg38 as the only protonatable residue (but with a high pK_a), sufficient H-bonding between residues and waters could be envisaged to provide a proton conducting pathway.

However, proton connectivity between this region and the N phase at Asp407 is more problematic. This lower region runs from Ser461 to Asp407 via His413. Only two water molecules are observed in the crystal structures immediately above and below His413, which

is insufficient to form a proton conducting pathway. In oxidised CcO Ser382 makes an H-bond to the –OH group of the hydroxyethylfarnesyl chain of heme *a*. However, in fully reduced CcO this bond breaks and Ser382 moves into the helix X (10). This has been proposed to form a cavity that hydrates this region sufficiently to enable proton transfer. In the simulations Ser382 does undergo a conformational change that is in agreement with the structural data. However, its dissociation from the hydroxyethylfarnesyl group does not induce the postulated bridging of the gap between His413 and Ser461 with additional waters. Indeed, the same type of structural change of the equivalent serine has been observed in *Rhodobacter sphaeroides* CcO where an H channel is not operative (33), and in which recent kinetic data on wild-type and a Ser425Ala mutant show no major differences in their reaction kinetics or coupling efficiencies (50). It is not conserved in the structurally and biochemically well-characterized B-type oxidase from *Thermus thermophilus*, the quinol oxidase from *Escherichia coli* and various C-type oxidases (57). Hence, its conformational change may be more associated with properties or stability of the heme *a* rather than having a channel-opening function.

Our simulations show that this lack of water-based connectivity in the region around His413 persists, as long as the latter is neutral, which is its calculated protonation state. Only when simulations are performed with protonated His413 does rapid hydration and transient water wire formation occur in the lower part of the H channel. However, the pK_a of this His is likely to be too low to be able to form its imidazolium state, even when heme *a* becomes reduced. Firstly, its environment is rather nonpolar (Fig. S2). Secondly, continuum electrostatic calculations indicate a very low pK_a, even when heme *a* is reduced. Thirdly, the weak E_m/pH dependency of heme *a* (51, 52) shows that no single residue has a strongly linked redox Bohr protonation. Therefore, since the neutral protonation state is very likely the physiologically relevant state, we conclude that the absence of sufficient water density in the lower part of the channel remains the major concern in models in which the structure functions in conducting pumped protons across the structure. It might also be noted that His413 is not well conserved in eukaryotic CcOs, being absent for example from yeast and many insect and worm species, highlighting the notion that, if indeed the H channel is operative as a proton channel, this function is unlikely to be widespread in eukaryotes. Indeed, direct tests with mutants of *S. cerevisiae* CcO have shown that it does not function in this way in this enzyme (11, 37, 38).

Overall, the data presented here are unable to support the viewpoint that H channel is a proton pumping route in bovine CcO unless the unlikely protonation of His413 can be

demonstrated. An alternative (or perhaps in bovine CcO an additional) role for the H channel in all A-type CcOs as a dielectric well of channel was suggested (13). In such a role, the function of the more polar residues is to adjust their dipoles in response to the change of charge on the buried heme when it changes redox state, since charge neutralisation is not achieved with a redox Bohr protonation. Some evidence that this is occurring to some extent comes here from the calculated overall electric dipole moment of the residues surrounding heme *a*. Their polarity increases on oxidation of heme *a*, consistent with the net +1 charge of this state. Whether the polarity of these groups could also be altered by allosteric effects of supernumerary subunits or by ligand binding, hence providing a means for external factors to modulate internal kinetic properties, remains to be tested experimentally.

Models and Methods

Model system construction

Three different model systems (C1, C2 and C3) were constructed from the oxidised bovine CcO crystal structure (PDB id: 1V54) (31) in order to perform classical MD simulations. In model system C1, the entire 13 subunit monomeric CcO was immersed in a lipid bilayer formed by 50% phosphatidylcholine (DLPC 12:0/12:0), 30% phosphatidylethanolamine (DLPE, 12:0/12:0) and 20% phosphatidylinositol (DMPI 14:0/14:0), which was then solvated with TIP3 water molecules, and K⁺ and Cl⁻ ions to account for 100 mM ionic strength. The entire membrane-protein-solvent system was constructed by using CHARMM-GUI tools (58, 59) and consisted of ~390000 atoms. A second smaller model system (C2) comprising ~280000 atoms was also constructed to study different intermediates of the catalytic cycle of CcO, and to test the consistency of results in comparison to the large model system C1. C2 consisted of the three core catalytic subunits (I, II and III) immersed in a hybrid lipid bilayer formed by dianionic cardiolipin (CL, 18:2/18:2/18:2/18:2), phosphatidylcholine (PC, 18:2/18:2), and phosphatidylethanolamine (PE, 18:2/18:2), in a ratio of 1: 3.38: 3.05, which mimics the composition of the inner mitochondrial membrane (see also ref. (17)). Water (TIP3) was added as a solvent, and 100 mM ionic strength was established with Na⁺ and Cl⁻ ions. The third model system (C3 comprising ca. 130000 atoms) was also constructed to study long timescale behaviour of hydration in the lower part of the H channel. For this, a two subunit enzyme was immersed in a hybrid lipid bilayer (same as C2), and two independent simulations of up to 1 μ s each, as well as multiple short simulations of 15-30 ns, were performed in the fully reduced state of the enzyme (see Table 3).

Fully atomistic classical MD simulations were performed in different

redox/protonation states, as described in Tables 1 (C1), 2 (C2) and 3 (C3). All internal water molecules present in the 1V54 atomic structure were retained. Standard protonation states at pH 7 were used for all titratable residues except for those listed in Tables 1-3. All simulations were performed using NAMD (60) software together with the CHARMM force field for protein, lipids, water and ions (61, 62). Parameters for a classical H_3O^+ ion were taken from an earlier study of Sagnella and Voth (63). The parameters for metal centers were obtained from the earlier study by Johansson et al. (64). MD simulations were carried out at physiological temperature and pressure using 1 or 2 fs time steps for systems C2 and C1/C3, respectively. The PME method (65) as implemented in NAMD was used to treat long-range electrostatics. The simulation trajectory data was saved every 10 or 100 ps, and Visual Molecular Dynamics (VMD) software was used for trajectory visualization and analyses (66).

The C1 system in state I (oxidised, Table 1) was equilibrated for 100 ns, and the resulting structure was used to initiate C1 II-VI simulations. In these further states, the protonation states of functionally important residues and/or the protonation pattern within the BNC were altered. The total simulation time for all C1 systems was ca. 1.3 μs . In the smaller C2 model systems, both redox (OX – oxidised, RED - reduced) states of metals and protonation states of key amino acids were varied, with particular focus on the influence on H channel structure of electron transfer from Cu_A to heme *a* (C2-II versus C2-I) and from heme *a* to a BNC intermediate (C2-II versus C2-III), and of reduction of heme *a*/ Cu_A (C2-VII versus C2-VIII). The total simulation time of all C2 systems was ca. 2.2 μs and for C3 ~2.3 μs .

Continuum electrostatic calculations were also performed on snapshots obtained from MD simulations of system C2 to estimate pK_a s. Up to 100 simulation snapshots were chosen to calculate the pK_a s in different redox/protonation states of the enzyme. The MEAD program (67) was used to calculate intrinsic pK_a and site-site interaction energies, and KARLSBERG software (68) to estimate the final pK_a of the residue by a full titration calculation of all titratable sites in a three subunit enzyme using reference pK_a values for Arg, Asp, Glu, His, Lys, Tyr and propionate groups of 12.0, 4.5, 4.6, 6.2, 10.4, 9.7 and 4.8, respectively. In order to further validate the obtained pK_a values, we also performed pK_a estimation by using Propka (69, 70) and Alchemical (71) free energy simulations, as implemented in NAMD, using a small model system containing only subunit I. Regardless of the method used, we consistently obtained low pK_a values for His413 ($\text{pK}_a < 5$).

Six QM/MM MD simulations were performed using Turbomole and Amber packages (72-74). The QM region consisted of His413, Asp407 and water molecules around these

residues (see Videos S1 and S2). Link atoms (hydrogens) were introduced between C α and C β atoms of His413 as well as Asp407. An extra proton was modeled on His413, on Asp407 or next to the water molecules in the QM region (ca. 8.5, 6.2, 2.9, and 2.3 Å from Asp407), followed by a 1000 step conjugate gradient geometry optimization, and molecular dynamics at 310 K (Langevin thermostat) for 5-15 ps using 1 fs time step.

System stability

Analysis of simulation trajectories reveal that C1 large model systems stabilized with a root mean square deviation (RMSD) plateaued in the range of 2 – 3 Å (Fig. S7). For C2 systems, RMSD stabilized at 3 – 4 Å (Fig. S8). These data suggest that simulations had converged sufficiently to be able to observe any rapid dynamics of water molecules in the H channel.

Simulations on model system C1 showed no large scale conformational changes in the residues involved in the H channel induced by variations in possible ground state of the oxidised BNC or by changes in the protonation states of the selected amino acid residues. When final resulting structures of C1 I-VI simulations were compared for the positions of key H channel residues (Arg38, Asp51, Tyr54, Tyr371, His413, Thr424, Tyr440, Tyr441, Ser454, Ser458, and Ser461), the RMSD of all non-hydrogen atoms was smaller than 0.95 Å, and their positions were remarkably similar to those in the crystal structure. A similar scenario was observed when final snapshots of C2 simulations were compared. The RMSD for all C2 simulations was \leq 0.9 Å, except for the system C2-II (RMSD of 1.02 Å). In spite of the differences in system sizes and lipid compositions of model systems C1 and C2, the structures obtained from simulations are remarkably similar (Fig. S1).

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Figure legends

Fig.1. Structure of bovine CcO and key features of the H channel. A:- The 13 subunit bovine CcO is shown in its approximate position embedded in a lipid bilayer. Subunits I, II and III are in green, yellow and pink, respectively, with additional subunits in blue. B:- Details of the H channel. In the text, residues that are components of the putative H channel are divided into TOP (Y54, Y371, Y440, S441; shown in blue); MIDDLE (R38, T424, S454, S458; shown in cyan; LOWER (H413, S461; shown in green). Other residues discussed in the text are D51 at the P phase interface, its H-bonded partner S205 of subunit II, D407 at the N phase and S382 of helix X (see text for details). The inset shows a reoriented view to illustrate the H bond between S382 and the hydroxyethylfarnesyl chain of heme *a*. Water molecules within the H channel are shown as red spheres. Additional waters within 5 Å of D51 or S205_{II} at the P phase, or within 5 Å of D407 at the N phase, are shown as blue spheres. Drawn from coordinates of oxidised bovine CcO in PDB 1V54 (31).

Fig. 2. Time dependencies of numbers of water molecules adjacent to heme *a*. Only water molecules within 4 Å of the N and O atoms of the side chains of residue 461, 382, 458, 424, 38, 454, 371, 451, 54 and 428 are included. The plot shown is a running average of 100 simulation snapshots taken at 100 ps time intervals.

Fig. 3. Occupancy of water molecules in the neighborhood of His413 and heme *a* (yellow) from simulations C1-I (A), C1-VI (B), C2-II (C) and C2-IV (D). Occupancy is displayed as an isosurface with isovalues of 0.08 (A and B) or 0.10 (C and D). Instantaneous positions of water molecules are shown as purple spheres, polar amino acid residues in green and basic residues in blue. Hydrogens are not shown for clarity.

Figure 4. Time dependencies of Ser382 conformation and occupancy of water molecules. Ser382 conformation is represented by the H-bond between the sidechain O atom of Ser382 and the O atom of the hydroxyethylfarnesyl group of heme *a* (black trace, see also Fig. 1B). The numbers of water molecules within 6 Å of the latter are also plotted (red trace).

Figure 5. Time dependencies of occupancy of water molecules in the neighborhood of

His413. The plot shown is a running average of 100 simulation snapshots taken at 100 ps time intervals. Only water molecules whose oxygen atoms are within 4 Å of the nitrogen atoms of His413 sidechain are considered.

Supplementary Material

Insights into functions of the H channel of cytochrome *c* oxidase from atomistic molecular dynamics simulations

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Figure S1: Comparison between C1 and C2 simulations. Snapshots from C1-VI (brown) and C2-IV (green) are compared. When the C α of both subunits I (shown as cartoons) are aligned, an RMSD of 2.2 Å is obtained, which is reduced to 1.1 Å when the loops are not considered. Positions of H channel residues are shown as sticks and are very similar (RMSD of 1.2 Å, or 0.8 Å if only the backbone is considered).

Figure S2: Predominantly hydrophobic environment around H413. Sidechains of the residues around H413 are shown as sticks and water molecules are shown as red spheres. Top panel: Crystal structure (PDB 1V54). Middle panel: After 300 ns (setup C1-I) with H413 in its neutral form. Lower panel: After 150 ns (setup C1-VI) with H413 in its cationic (+1) form. Protonation of H413 attracts water molecules to the H channel (see main text). The pocket occupied by H413 and the surrounding water molecules is shown as a surface.

Figure S3. Number of water molecules (oxygen atoms) within 8 Å of His413 from simulations C3-II (five independent runs). All simulations started from a fully hydrated lower

region of the H channel.

Figure S4. Number of water molecules (oxygen atoms) within 8 Å of His413 from simulations of C1-I (three independent runs). All simulations started from a fully hydrated lower region of the H channel. For clarity, plots display a running average of 20 snapshots.

Figure S5. Free energy profiles for the hydration of lower region of H channel when His413 is neutral (top panel) or protonated (bottom panel). As reaction coordinate we have used $\langle r_{\text{wat-H413}} \rangle$, the average distance between the imidazole ring of His413 and the center-of-mass of the 10 water molecules closest to His413. The results for the protonated His413 (C1-VI simulations, red) show a single minimum at $\langle r_{\text{wat-H413}} \rangle = 5.5$ Å, representing a stable water wire. Deprotonation of His413 (C1-I, blue) changes the free energy landscape, and the water wire is no longer stable. Minima are found instead between $\langle r_{\text{wat-H413}} \rangle = 9-11$ Å, which are not compatible with a water wire connecting the bulk and the lower part of the H channel. Notice that the zero of the energy scale correspond to the most stable conformation for each of the systems.

Figure S6: Snapshots at different time intervals display the fluctuation of electric dipole moment from simulations C2-I (red) and C2-II (blue). The amino acid side chains (yellow for C2-I, and brown for C2-II) are shown (see also Table 6 in main text). Backbone is displayed as white transparent ribbon, and low-spin heme is in licorice representation (green for C2-I and purple for C2-II).

Figure S7: Root mean square deviation (RMSD) in Å vs simulation time (in ns) for C1 simulations. The structural alignment to zeroth simulation frame and RMSD calculations were performed using C α atoms.

Figure S8: RMSD (in Å) vs simulation time (in ns) for C2 simulations. The structural alignment to zeroth simulation frame and RMSD calculation were performed using C α atoms. Only the first replicas of setups C2 I, II, VII and VIII were used for RMSD calculations.

Video S1: A ~5 ps QM/MM MD trajectory in which a proton is modeled next to a water molecule 6.2 Å from Asp407.

Video S2: A 15 ps QM/MM MD trajectory in which Asp407 is initially protonated. The water wire between protonated Asp407 and neutral His413 dissociates in agreement with classical simulation data (see main text).

Still image for video S1.

Still image for video S2.